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(54) Title: GENE THERAPY VECTOR FOR THE TREATMENT OF LOW OR DEFECTIVE RED BLOOD CELL PRODUCTION

(57) Abstract

The present invention involves gene therapy for the enhancement of red blood cell production. The delivery and expression of the erythropoietin gene, elicits a stable increase in red blood cell production. The present invention includes recombinant delivery vectors, compositions, alternative gene therapy strategies, and transfected cells which express sufficient erythropoietin to present a physiologically significant systemic response.

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GENE THERAPY VECTOR FOR THE TREATMENT OF LOW OR DEFECTIVE RED BLOOD CELL PRODUCTION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a novel approach to the treatment of low or defective red blood cell production. In particular, the invention provides for the sustained systemic production of erythropoietin following the modification of target cells by gene transfer.

Description of the Background

Erythropoiesis, the production of red blood cells, occurs continuously to offset cell destruction. Erythropoiesis is a precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone, erythropoietin.

Erythropoietin is normally present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is sufficient to stimulate the replacement of red blood cells which are naturally lost through aging.

The amount of erythropoietin in the circulatory system is increased under conditions of hypoxia when oxygen transport by blood cells to tissue is reduced. Hypoxia may be caused by loss of large amounts of blood due to hemorrhage, destruction of red blood

cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, erythropoietin increases red blood cell production by stimulating the conversion of precursor cells in the bone marrow into erythroblasts. The erythroblasts subsequently mature, synthesize hemoglobin and are released into the circulatory system as red blood cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, erythropoietin in circulation is decreased.

Because erythropoietin is essential in the process of red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. While the injection of recombinantly produced human erythropoietin is a proven therapy for the treatment of blood disorders, it would be advantageous to enhance the endogenous production of erythropoietin in a patient or mammalian subject.

SUMMARY OF THE INVENTION

The present invention provides, for the first time, the successful development of a method for the enhancement of red blood cell production by gene therapy. The invention demonstrates that expression vectors can be constructed using an expression control sequence and an erythropoietin gene, operatively linked to the control sequence and capable of expression in transfected target cells, wherein the nucleic acid construct is capable of eliciting the

expression of erythropoietin sufficient to increase red blood cell production.

Having elucidated the means for the enhancement of red blood cell production by increasing the presence of erythropoietin *in vivo*, the present invention supports the development of gene therapy techniques for the treatment of low or defective red blood cell production. Also comprehended by the invention are pharmaceutical compositions involving effective amounts of the nucleic acid constructs together with a pharmaceutically acceptable delivery vehicle including suitable diluents, buffers and adjuvants. The compositions can further include a carrier capable of promoting target cell uptake of the nucleic acid constructs. Such carriers include liposomes, protein complexes and viral carriers suitable for gene transfer techniques.

We have found no previous report of the use of target cells for the purpose of erythropoietin gene transfer and subsequent *in vivo* production of recombinant erythropoietin with a demonstrated pharmacological response. In a specific embodiment, the invention involves the development of myoblast-mediated gene therapy for the *in vivo* production of erythropoietin. The invention further describes the use of expression vectors involving non-specific and muscle-specific promoters, and the suitability of such vectors for generating stable myogenic cell lines which, following introduction into skeletal muscle, can elicit sufficient production and secretion of erythropoietin to present a physiologically significant systemic response.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a DNA sequence for erythropoietin.

DETAILED DESCRIPTION OF THE INVENTION

Gene therapy for anemia comprises the delivery of a gene for erythropoietin to cells, either *in vivo* or *in vitro*. Delivery and expression of the gene results in the production of erythropoietin in a physiologically-functional amount sufficient to increase red blood cell production. The erythropoietin gene used in the present invention, is a nucleic acid sequence which encodes a functional erythropoietin protein. Thus, variations in the actual sequence of the gene can be tolerated provided that functional erythropoietin is expressed. An erythropoietin gene used in the practice of the present invention can be obtained through conventional methods such as DNA cloning, artificial construction or other means.

Gene transfer of the erythropoietin gene in accordance with the present invention can be accomplished by any suitable gene therapy technique involving a nucleic acid construct or recombinant vector containing a DNA sequence that encodes erythropoietin. The nucleic acid constructs generally will be provided as an expression cassette or expression control system which will include as operatively linked components in the direction of transcription, a transcriptional initiation region, the erythropoietin nucleic acid sequence of interest and a transcriptional termination region wherein the transcriptional regulatory regions are functional in a mammalian host. It may be preferred that a

recombinant vector construct not become integrated into the host cell genome of the patient or mammalian subject, and therefore, it may be introduced into the host as part of a non-integrating nucleic acid construct. A coding sequence is "operatively linked to" or "under the control of" the expression control system in a cell when DNA polymerase will bind the promoter sequence and transcribe the erythropoietin-encoding sequence into mRNA. Thus, the nucleic acid construct includes a DNA sequence which encodes a polypeptide directly responsible for a therapeutic effect, as well as a sequence(s) controlling the expression of the polypeptide.

The nucleic acid constructs in the invention include several forms, depending upon the intended use of the construct. The transcriptional and translational initiation region (also herein referred to as a "promoter"), preferably comprises a transcriptional initiation regulatory region and a translational initiation regulatory region of untranslated 5' sequences. In alternate embodiments, the promoter may be modified by the addition of sequences, such as enhancers, or deletions of nonessential and/or undesired sequences. The promoter will have a DNA sequence sufficiently similar to that of a native promoter to provide for the desired specificity of transcription of the erythropoietin DNA sequence. The promoter may include natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. It will also be appreciated by those skilled in the art that the expression control sequence may contain a suppresser sequence to regulate the expression of erythropoietin.

For the transcriptional initiation region, or promoter element, any region may be used with the

proviso that it provides the desired level of transcription of the erythropoietin nucleic acid sequence. The transcriptional initiation region may be native to or homologous to the host cell, and/or to the DNA sequence to be transcribed, or foreign or heterologous to the host cell and/or the DNA sequence to be transcribed. By foreign to the host cell is intended that the transcriptional initiation region is not found in the host into which the construct comprising the transcriptional initiation region is to be inserted. By foreign to the DNA sequence is intended a transcriptional initiation region that is not normally associated with the DNA sequence of interest. Efficient promoter elements for transcription initiation include the SV40 (simian virus 40) early promoter, the RSV (Rous sarcoma virus) promoter, the Adenovirus major late promoter and the human CMV (cytomegalovirus) promoter.

Inducible promoters also find use with the expression control sequences where it is desired to control the timing of transcription. Examples of promoters include those obtained from a β-interferon gene or those obtained from steroid hormone-responsive genes. Such inducible promoters can be used to regulate transcription of the transgene by the use of external stimuli such as interferon or glucocorticoids. Because the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements also can be used. Tandem arrays of two or more inducible promoter elements may increase the level of induction above baseline levels of transcription which can be achieved when compared to the level of induction above baseline achieved with a single inducible element.

Transcriptional enhancer elements may also be included in the expression control sequence. The term "transcriptional enhancer elements" includes DNA sequences which are primary regulators of transcriptional activity and which can act to increase transcription from a promoter element. The combination of promoter and enhancer element(s) used in a particular expression cassette can be selected by one skilled in the art to maximize specific effects. Different enhancer elements can be used to produce a desired level of transgene expression in a wide variety of tissue and cell types. For example, the human CMV immediate early promoter-enhancer element can be used to produce high level transgene expression *in vivo*.

Examples of other enhancer elements which confer a high level of transcription on linked genes in a number of different cell types from many species include enhancers from SV40 and RSV-LTR. The SV40 and RSV-LTR are essentially constitutive. They may be combined with other enhancers which have specific effects, or the specific enhancers may be used alone. Thus, where specific control of transcription is desired, efficient enhancer elements that are active only in a tissue-, developmental-, or cell-specific fashion are of interest.

Tandem repeats of two or more enhancer elements or combinations of enhancer elements may significantly increase erythropoietin expression when compared to the use of a single copy of an enhancer element. Enhancer elements from the same or different sources flanking or within a single promoter can in some cases produce transgene expression in each tissue in which each individual enhancer acting alone would have an effect, thereby increasing the number of tissues in which transcription is obtained. In other cases, the

presence of two different enhancer elements results in silencing of the enhancer effects. Evaluation of particular combinations of enhancer elements for a particular desired effect or tissue of expression is within the level of skill in the art.

Gene transfer procedures are known to those skilled in the art and include cell transformation using calcium phosphate coprecipitation, lipofection of the target cells with liposome/gene or lipid/gene conjugates, plasmid-mediated transfer, DNA protein complex-mediated transfer and viral vector-mediated transfer. Viral vector transfer can include suitable techniques such as transfer by recombinant retroviral vectors, adenovirus vectors and adeno-associated virus vectors. Thus, the present invention includes the use of carriers to facilitate gene transfer, and different carriers may be selected as appropriate to optimize transfer to the desired cell-type which is targeted for vector delivery. It will also be appreciated that the various carriers may be selected or modified for preferential uptake by the cell-type which is targeted for vector delivery. For example, the carrier can include a selected ligand to effectively target the cells of interest. In addition, the vector may contain one or more targeting sequences, generally located at both ends of the exogenous DNA sequence to be expressed. Such a construct is useful to integrate exogenous DNA into the target cell.

The cells targeted for gene transfer in accordance with the present invention include any cells to which delivery of the erythropoietin gene is desired. While a variety of cells may be transfected, it was determined that muscle cells are especially appropriate targets for gene transfer and the expression of physiologically active amounts of

erythropoietin. For the purposes of the present invention, a physiologically active or acceptable level of erythropoietin gene function refers to a level of *in vivo* erythropoietin manufacture and function sufficient to cause an increase in red blood cell production. Increased red blood cell production can be readily determined by an appropriate indicator such as detection of changes in hematocrit levels. The level of erythropoietin gene function sufficient to cause an increase in red blood cell production can readily be determined by a comparison of pretreatment or baseline hematocrit level to the post-treatment hematocrit level.

Cells or cell populations can be treated in accordance with the present invention either *in vivo* or *in vitro*. For example, in *in vivo* treatments, recombinant erythropoietin vectors can be administered to the patient, preferably in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. The dosages administered can vary from patient to patient and will be determined by the level of enhancement of erythropoietin function balanced against any risk of side effects. Monitoring levels of transduction, erythropoietin expression and/or the levels of red blood cells will assist in selecting and adjusting the dosages administered.

In vitro transduction is also contemplated within the present invention. Cell populations can be removed from the patient, or otherwise be provided, transfected with the erythropoietin gene in accordance with the present invention, and then administered to the patient. The transfected target cells may be reintroduced by any suitable means, such as injection or implantation, and the cells will typically be delivered to target tissue of the same cell type as the target cells. For example, muscle cells may serve

as the target cells. Myoblasts can be isolated and manipulated *in vitro*, transfected with the erythropoietin vector, and the transformed cells are then reintroduced into muscle tissue. The unique biology of muscle cells allows the transfected cells to form new myofibers or fuse into old ones. It was discovered that the transplanted nuclei are sustained and active for prolonged periods of time in a normal, multinucleated environment with little or no nuclear replication for up to six months. Moreover, it was discovered that the muscle cells will sustain the production and secretion of the erythropoietin protein sufficient to result in increased red blood cell production.

The present invention is also amenable to the use of homologous recombination genome-modification methods. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes (Kucherlapati, *Prog. in Nucl. Acid Res. and Mol. Biol.* 36:301 (1989)). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al., *Cell*. 44:419-428, 1986; Thomas and Capecchi, *Cell*. 51:503-512, 1987; Doetschman et al., *Proc. Natl. Acad. Sci*. 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman et al., *Nature*. 330:576-578, 1987).

Through homologous recombination, a piece of DNA that one desires to insert into the genome can be directed to a specific region of the gene of interest by attaching it to "targeting DNA". "Targeting DNA" is DNA that is complementary (homologous) to a region of the genomic DNA. When two homologous pieces of single stranded DNA (i.e., the targeting DNA and the genomic DNA) are in close proximity, they will

hybridize to form a double stranded helix. Attached to the targeting DNA is the DNA sequence that is to be inserted into the genome.

Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. It is a general property of DNA that has been inserted into a cell to hybridize and therefore recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence of DNA, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transfered DNA is incorporated into the genome.

If the sequence of a particular gene is known, a piece of DNA that is complementary to a selected region of the gene can be synthesized or otherwise obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous region within the genome. If this hybridization occurs during DNA replication, this piece of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be backstitched into the newly synthesized daughter strand of DNA.

In the present invention, attached to these pieces of targeting DNA are regions of DNA will interact with the nuclear regulatory proteins present within the cell and, optionally, amplifiable and selectable DNA markers. Thus, the expression of erythropoietin may be achieved not by transfection of

DNA that encodes the erythropoietin gene itself, but rather by the use of targeting DNA (regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous erythropoietin gene with recognizable signals for transcription. With this technology, it is possible to express and to amplify any cognate gene present within a cell type without actually transfecting that gene. In addition, the expression of this gene is controlled by the entire genomic DNA rather than portions of the gene or the cDNA, thus improving the rate of transcription and efficiency of mRNA processing. Furthermore, the expression characteristics of any cognate gene present within a cell type can be modified by appropriate insertion of DNA regulatory segments and without inserting entire coding portions of the gene of interest.

In accordance with the present invention, homologous recombination provides new methods for expressing a normally transcriptionally silent erythropoietin gene, or for modifying the expression of an endogenously expressing gene. The erythropoietin gene will be provided with the necessary cell-specific DNA sequences (regulatory and/or amplification segments) to direct or modify expression of the gene within the muscle cell. The resulting DNA will comprise the DNA sequence coding for erythropoietin directly linked in an operative way to heterologous (for the cognate DNA sequence) regulatory and/or amplification segments. A positive selectable marker is optionally included within the construction to facilitate the screening of resultant cells. The use of the neomycin resistance gene is preferred, although any selectable marker may be employed. Negative selectable markers may, optionally, also be employed. For instance, the

Herpes Simplex Virus thymidine kinase (HSVtk) gene may be used as a marker to select against randomly integrated vector DNA. The fused DNAs, or existing expressing DNAs, can be amplified if the targeting DNA is linked to an amplifiable marker.

In the specific examples which follow, a myoblast cell line was established which stably expressed the human erythropoietin gene. The cell line was established by transfecting the cells with a plasmid containing the erythropoietin gene driven by a CMV promoter. The plasmid was derived from pCD vector 1 (Okayama et al., A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Bio.* 3:280-289, 1993) as described in the following examples. Genes encoding erythropoietin are described in United States Patent Number 4,703,008 issued October 27, 1987, and entitled DNA Sequences Encoding Erythropoietin, and Figure 1a-d. The plasmid included a gene for neomycin resistance such that transformed cells could be selected by antibiotic resistance. After the expansion of 23 randomly selected clones, the clones were screened for the secretion of erythropoietin into the culture media by Western blot and radioimmunoassay.

Table 1
Erythropoietin (EPO) Expression in Transformed Myoblasts as Determined by RIA

C2 cell clone	Erythropoietin (units/ml)
60k, EPO2, #6, 1/14	4.9
60k, EPO1, #5, 1/11	28.7
30k, 7, 1/11	0.16
60k, EPO1-2, #1, 1/11	11.8
60k, EPO1, #4, 1/19	2.37
60k, 3a, #6, 1/11	1.27
120k, 4, 1/11	0.38
60k, EPO1-1, #1, 1/11	2.6
60k, EPO1, #10, 1/11	2.17
60k-2, EPO1, #10, 1/11	22.8
60k-2, EPO1, #8, 1/11	2.3
60k, EPO1, #8, 1/11	0.18

As illustrated in Table 1, twelve clones had measurable erythropoietin production. The assay involved a typical RIA procedure and was performed substantially in accordance with the method described by Egrie et al., *Journal of Immunological Methods*, 99:235-241 (1987). Values for erythropoietin levels in the media of the positive clones ranged from 0.18 to 28 Units per ml. This represented a production/secretion range of approximately 2×10^{-8} to as much as 5×10^{-7} Units per cell per hour (assuming linear synthesis and secretion and no significant decay of activity).

The present invention demonstrates the efficacy of gene transfer to obtain sustained *in vivo* production of a therapeutic polypeptide, such as

erythropoietin, at levels sufficient to enhance red blood cell production. It will be appreciated by those skilled in the art that refinements in the selection of promoter and enhancer genes will serve to optimize the expression of erythropoietin in the transfected target cells. For example, it is also within the present invention to use muscle-specific expression control sequences for high level recombinant protein expression in transformed muscle cells. High activity promoter and enhancer cassettes can be used to intensify recombinant gene expression. Such promoters will increase the levels of therapeutic recombinant proteins synthesized and secreted by both newly formed myofibers as well as muscle fibers that contain a mixture of donor and recipient myonuclei.

The major contractile proteins of thin and thick filaments (e.g., alpha-actins, troponin C, myosin heavy chains, as well as several muscle enriched enzymes, such as creatine kinase and carbonic anhydrase III) all have genes that are expressed at high levels in muscle. Promoters and enhancers of most of these genes have been the subjects of intense investigation and analysis (reviewed in Bishopric, et al., The molecular biology of cardiac myocyte hypertrophy p. 399-412. In L.H. Kedes and F.E. Stockdale [ed.]. *Cellular and Molecular Biology of Muscle Development*. vol. 93, Alan R. Liss, Inc. New York 1986; and Wade, et al., Developmental regulation of contractile protein genes p. 179-188. [ed.], *Annu. Rev. Physiol*. vol. 51, Annual Reviews, Inc. Palo Alto, 1989). Muscle-specific gene expression is usually associated with muscle-specific transcription factors including members of the MyoD family (Weintraub, et al., The *myoD* gene family: nodal point during specification of the muscle cell lineage. *Science*. 251:761-766, 1991) and the MEF-2 site binding

factors (Cserjesi, P. and E.N. Olson. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Molecular And Cellular Biology*. 11:4854-62, 1991; and Olson, et al., Molecular control of myogenesis: antagonism between growth and differentiation. *Mol. Cell. Biochem*. 104:1-2, 1991.). The appropriate selection and combination of vector elements will provide for optimal regulation of muscle cell gene expression and sustained high levels of expression of recombinant genes introduced into muscle cells.

A number of muscle-specific genes have been cloned, and the promoters analyzed: these include skeletal actin, cardiac actin, Troponin C fast, Troponin C slow and Troponin I slow, as well as beta and gamma cytoskeletal actins. Other muscle specific promoters that have been the subject of detailed analysis include creatine kinase, myosin light chains and various myosin heavy chain genes as well as Troponins I, T and C. Detailed analyses of such enhancer and promoter regions that provide muscle specificity are available, as illustrated by the following brief summary.

Skeletal α-actin: The tissue specific distal promoter of the human skeletal α-actin gene (-1282 to -708) induces transcription in myogenic cells approximately 10-fold and, with the most proximal promoter domain (-153 to -87), it synergistically increases transcription 100-fold (Muscat, et al., Multiple 5' flanking regions of the human skeletal actin gene synergistically modulate muscle specific gene expression. *Mol. Cell Biol*. 7:4089-4099, 1987). A short fragment of the distal promoter, the distal regulatory element (DRE) from -1282 to -1177, functions as a muscle-specific composite enhancer (Muscat, et al., The human skeletal α-actin gene is

regulated by a muscle-specific enhancer that binds three nuclear factors.

Cardiac α-actin: The cardiac α-actin gene is the fetal isoform of α-actin in rodents and human muscle, but it does not express after birth in rodents. The down regulation appears to be dependent on nucleotide sequences far downstream of the transcribed gene. In adult human muscle, cardiac α-actin represents about 5% of the α-actin mRNA. The cardiac α-actin promoter and endogenous gene are highly expressed in cell lines derived from skeletal muscle. Thus, the cardiac actin gene promoter and upstream elements are candidate elements as positive regulators of muscle specific gene expression in skeletal muscle cells.

Skeletal Fast-twitch Troponin C gene: The expression of the human fast-twitch skeletal muscle troponin C (TnC or TnCfast) gene is muscle-specific and confined to the class of fast-twitch myofibers in adult skeletal muscle. There is a strong classical enhancer element within the 5'-flanking sequence of this gene which is required for the transcriptional activity. A MEF-2 site alone in this enhancer is sufficient to support high level transcription. Interestingly, and unlike enhancers of other muscle genes, the human fast TnC enhancer is muscle cell specific, but only if linked to its own basal promoter which is itself not muscle cell restricted. This suggests that interactions between the enhancer and the basal promoter of the human fast TnC gene are responsible for its muscle restricted expression.

Slow-Twitch/cardiac Troponin C gene: At least four separate elements cooperate to confer muscle specific expression on the human slow twitch skeletal/cardiac troponin C (HcTnC or TnCslow) gene: a basal promoter (from -61 to -13) augments

transcription 9-fold, upstream major regulatory sequences (from -64 to -1318 and from -1318 to -4500) augment transcription 18-fold and 39-fold, respectively, and a position and orientation independent enhancer in the first intron (from +58 to +1519) augments transcription 5-fold. This enhancer increases muscle specific CAT activity when linked to its own promoter elements or to a heterologous SV40 promoter, and the effects appear to be multiplicative rather than additive. When the various promoter/CAT chimeric plasmids are cotransfected with a MyoD expression vector into 10 T 1/2 cells, constructs carrying either the TnC promoter or the first intron of the gene are >500-fold induced. Thus, each of these regulatory regions is capable of responding directly or indirectly to the myogenic determination factor, MyoD. These observations suggest that skeletal muscle expression of the HcTnC gene is cooperatively regulated by the complex interactions of multiple regulatory elements.

Slow-twitch Troponin I gene: At least three separate elements spaced over 1 kb of the 5' upstream regions of the human slow twitch troponin I gene (HsTnI) combine to synergistically regulate muscle specific gene expression. A basal promoter lies within 300 base pairs of the transcription start site and two independent muscle specific enhancers 800 and 1000 base pairs upstream. All three appear to be required for expression. These observations suggest that muscle expression of the HsTnI gene is cooperatively regulated by the complex interactions of multiple regulatory elements.

cDNAs encoding the erythropoietin gene may be cloned into a variety of in-frame expression vectors. A non-muscle-specific beta-actin promoter, constructed

as a high level expression vector with neomycin selection capacity, contains the promoter and first intron of the human β-actin gene, a neomycin resistance gene, a bacterial origin and the SV40 late region polyadenylation signal (Gunning, et al., A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA.* 84:4831-5; 1987). The use of such a construct fosters high level transcription of inserted sequences in mammalian cells.

The erythropoietin sequence may also be cloned into an internally deleted human skeletal α-actin gene promoter that carries high level muscle specific expression. This construct carries an upstream element (from -1282 to -1177) linked to its own promoter from -153. This enhancer/promoter combination may be inserted in the place of beta-actin sequences to create a new muscle specific expression vector with neomycin selectability (pHaSKApr-1-neo).

Typically, the plasmid vectors are sequenced after construction to insure in-frame accuracy. The plasmids may be co-transfected into C2 myogenic cells along with a β-galactosidase expression vector. Twenty four hours after infection, the cells may be split 1:20 into 60 mm dishes with DMEM containing 20% fetal calf serum (FCS) and 0.4 mg/ml of neomycin (Geneticin® G418; Gibco Laboratories, Grand Island, NY). After 14 days of selection, individual clones may be isolated and expanded in DMEM containing 20% FCS and 0.2 mg/ml of G418. Since the ability of transferred cells to differentiate into myotubes *in vivo* is a likely requirement for their stability and longevity *in situ*, the clones are tested for their ability both to differentiate into myotubes in DMEM supplemented with 2% horse serum and to express beta-galactosidase. The β-galactosidase expression serves

as a histological marker to monitor survival, as well as both macroscopic and microscopic location, of injected myoblasts at the conclusion of the studies.

Individual clones are expanded and the culture media tested for polypeptide production by Western blot and radioimmunoassay. High level expressing clones are selected for further analysis.

Novel combinations of muscle-specific enhancer and promoter elements may be constructed and tested for increased polypeptide expression *in vitro*. The creation of myogenic cells expressing exceptionally high levels of recombinant polypeptides provides a means of reducing both the numbers of primary cells required for *ex vivo* manipulation and the numbers of cells required for gene therapy muscle cell transplant.

As described above, there are a number of strong muscle specific promoter and enhancer elements in the genes for contractile proteins. Plasmid expression vectors may be constructed from several of these components linked together. For example, a construct may include the parent skeletal actin chloramphenicol acetyltransferase (CAT) expression vector containing the upstream enhancer and the muscle specific promoter. To this may be added single copies of the TnCfast upstream enhancer, the TnCslow first intron enhancer element, and the MCK enhancer (Johnson, et al., Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. *Mol. Cell. Biol.* 9:3393-3399; 1989.). One or more of these elements may be added as 3-5 multimers. After checking the validity of the constructs by DNA sequencing, they may be used in calcium phosphate mediated gene transfer for transient transfection CAT assays by standard techniques. The plasmids may be cotransfected into C2 cells with RSV-

luciferase as a positive control standard in the search for promoters with heightened transcriptional activity. The plasmids may also be transfected into non-muscle (Hela or CV1) cells to evaluate their degree of muscle specificity. Beta-actin and RSV CAT constructs may be transfected into the same cells in parallel to serve as comparisons. Once a combination of enhancer/promoter elements that significantly augment transcription is identified, the regulatory region is transferred to replace the promoter in the neomycin vector (pHaSKApr-1-neo) described above along with a beta-galactosidase expression vector.

The present invention is further described by the following specific examples, which are illustrative but non-limiting.

EXAMPLES

Example 1
Construction of Erythropoietin cDNA for expression

A polylinker was inserted in the unique PstI site of a pCD vector 1 (Okayama et al., A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Bio.* 3:280-289, 1993) to generate the V19 vector. The V19.1 vector was derived from the V19 vector by switching direction of Eco R I and Hind III sites in relation to the SV40 promoter (see Table 2). This vector was then digested with Eco R I and Hind III to which the Bst E II to Hind III fragment of EPO cDNA and a Bst E II-Eco R I linker (see Table 2) were ligated to form V19.1 EPO.

Table 2

Linker and Adapter Constructs

the adapter to construct V19 from pCD:

```
5' agctgaattctctagaaaagctt 3'
    ||||||||||||||||||||
3' cttaagagatcttttcgaattaa 5'
```

the Bst E II-Eco R I linker:

```
5' aattccccccccgtgtg 3'
    ||||||||||||
3' gggggggcacaccagtg 5'
```

EPO cDNA was isolated from the vector V19.1 as an Eco R I-Hind III fragment. The sticky ends were filled in using T4 DNA polymerase in the presence of deoxyribonucleotides. A human cytomegalovirus vector (CMV/RC; Invitrogen Corporation, San Diego, CA), was digested with the restriction enzyme Hind III and was blunted using T4 DNA polymerase. An erythropoietin cDNA fragment was ligated to the CMV/RC vector to form a pRC/CMV-huEPO expression construct. *E. coli* DH5 alpha competent cells were transformed with the pRC/CMV-huEPO plasmid. Plasmid DNA was isolated, sequenced and used for transfection of mammalian cells.

Example 2

Transfection and screening of clones

Mouse myogenic C2 cells (Yaffe and Saxel, A myogenic cell line with altered serum requirements for differentiation, *Differen.* 7:159-166; 1977; and Serial passaging and differentiation of myogenic cells isolated form dystrophic mouse muscle. *Nature.* 270:725-727; 1977) were cultured in growth medium

consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum, 0.5% chick embryo extract (Gibco Laboratories) and 100 µg/ml of kanamycin (Gibco Laboratories) in 10% CO_2. Subconfluent C2 myoblast cells, in 100 mm dishes, were split 1:4 the day before transfection.

C2 cells were transfected by the calcium phosphate precipitation method using the pRC/CMV-huEPO plasmid of Example 1. Transfection mixtures were prepared as follows: a solution of 250 mM $CaCl_2$ (0.5 ml) was added dropwise to 8 µg of DNA in 0.5 ml of 2 x *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered saline (42 mM HEPES [pH 7.05], 270 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose). This was done with constant mixing. The calcium phosphate-DNA precipitate was left for 20 minutes at room temperature after which it was added to the cells. The cells were incubated for 16 hours, washed with phosphate-buffered saline (PBS), and incubated in growth medium (10 ml of 10% fetal calf serum in DMEM) for 48 hours.

Three days after transfection, the cells were split at 1:10 and incubated for 12 hours. For neomycin resistance selection, G418 was added to the medium at a final concentration of 400 µg/ml. The cells were supplemented with fresh growth medium containing 400 µg/ml of G418 every three days. After two weeks of incubation, 23 colonies were selected, and expanded.

For detection of erythropoietin producing clones, each clone was cultured at 1 x 10^6/100 mm dish in growth medium overnight, and incubated in serum-free DMEM (3 ml) for three days. The culture medium from each clone was collected, and erythropoietin concentration was determined by radioimmunoassay. The clones were aliquoted and stored in liquid nitrogen.

Example 3
Myoblast Transplantation

The highest producing clone was cultured in 150 mm dishes in growth medium containing 200 µg/ml of G418. When the cells reached 80% confluence, they were trypsinized and collected in ice-cold PBS. Total cell number was determined by hemocytometer. Cells were rinsed once again with PBS to remove residual trypsin, pelleted, and resuspended in a small volume of PBS to a concentration of 1 x 10^8 cells/ml. We routinely collected 2-3 x 10^8 cells for each experiment. Cells were kept on ice until use to prevent aggregation.

C3H mice syngeneic to the C2 cell line and nude mice (both 6-8 week-old) were used for transplantation. Animals were anesthetized by intraperitoneal administration of a mixture of ketamine (30 mg/kg) and xylane (4 mg/kg). A total of 4 x 10^7 cells per mouse were injected percutaneously through a 27 gauge needle at 40 different sites (1 x 10^6 cells/10 µl/site) into skeletal muscle tissue of both hind limbs. As a control, the same number of parental C2 cells were transplanted in the same manner.

Example 4
Hematocrit Measurement

Hematocrit was measured by microhematocrit method. Under general anesthesia, a total amount of approximately 200 µl of blood was collected by a retroorbital approach into three heparinized capillary tubes. Blood collection was performed one week prior to transplantation, three days and one week after

transplantation, and weekly thereafter. Control animals showed that this amount of blood collection did not significantly affect basal hematocrit levels. On several occasions, the hematocrit was also measured using a Coulter counter which showed parallel results with micro-hematocrit method. After hematocrit measurement, the plasma was collected and stored at -20°C for huEPO concentration measurement.

Example 5

Cell monitoring

a) Transplantation of C2 cells expressing β-galactosidase

To monitor the fate of injected cells, another C2 cell line which stabily expressed β-galactosidase was established. The cells were selected by neomycin resistance, and several clones expressing β-galactosidase were collected. One clone was used for transplantation into right hind limb (2 x 10^7 cells/mouse).

b) β-galactosidase Assays

The mice were sacrificed by cervical dislocation for the histochemical detection of β-galactosidase expression. Skeletal muscle tissue was excised and frozen immediately on dry ice. The excised muscles were then sectioned with a freezing microtome. The 10 µm thick sections were attached to microscope slides, fixed in 0.25% glutaraldehyde for 10 minutes, washed in PBS for 10 minutes, and stained in PBS containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$. Sections were incubated at 37°C overnight, rinsed in PBS, mounted and studied under microscope.

c) Reverse transcription-polymerase chain reaction (RT-PCR)

To confirm *in vivo* huEPO gene expression, muscle tissue was excised from the mice injected with the huEPO expressing C2 cells. The excised muscles were frozen by liquid nitrogen and homogenized by Polytron homogenizer (Brinkman Instruments, Company, NY). Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. The RNA (70µg) was resuspended in 50 µl of Tris/EDTA (pH 7.4). To this was added 50 µl of Tris/EDTA (pH 7.4) containing 8 U of RNAse-free DNAseI (Boehringer Mannheim, Indianapolis, IN), 4 U of placental RNAse inhibitor (Promega, Madison, WI), 20 mM $MgCl_2$, and 2 mM dithiothreitol. The reaction was stopped by the addition of DNAse stopping mixture containing 50 mM EDTA, 1.5 M sodium acetate (pH 4.8) and 1% sodium dodecyl sulfate. The RNA was treated with phenol/chloroform, chloroform, and ethanol-precipitated. For reverse transcription reaction, approximately 5 µg of RNA and 4 pmol primer complimentary to the 3' untranslated region of huEPO RNA were incubated at 70°C, rapidly cooled on ice, and treated with 100 U of reverse transcriptase (Superscript; Gibco Laboratories). The obtained cDNA was amplified by known PCR methods using primers including the initiation and stop codons.

d) Results

Table 3 shows the time course of mean hematocrit change after the transplantation of C2 cells expressing huEPO gene. Hematocrit started to increase three days after the transplantation of 4 x 10^7 cells into C3H syngeneic mice (A in Table 3). The peak

hematocrit was achieved two weeks after the transplantation. Hematocrit declined gradually thereafter, becoming lower than the basal level after week 4.

RT-PCR showed persistent huEPO mRNA expression for at least one month in the injected muscle, but not in the muscle from uninjected left hind limb. Mice transplanted with parental C2 cells did not show significant hematocrit increase. In contrast to C3H mice, nude mice (B) showed significantly higher and more sustained hematocrit increase for at least two months. When half the number of cells (2×10^7) were injected, the net hematocrit increase was also approximately half of that observed with 4×10^7 cells, thereby indicating that huEPO production can be regulated by cell number.

The mice transplanted with the C2 cells expressing β-galactosidase showed positive myofibers over the entire injected sites three months later. The injected C2 cells appeared to fuse among themselves as well as with preexisting myofibers. No β-galactosidase positive myoblasts were observed.

Serum huEPO concentration as determined at several points after transplantation by either radioimmunoassay or bioassay using an erythropoietin-responsive human leukemic cell line (UT-7/EPO) showed significantly elevated erythropoietin concentrations ranging from 90 to 3500 mU/ml. Erythropoietin concentrations before transplantation in these mice were <25 mU/ml.

Table 3
Mean Hematocrits in C3H Mice(A, n=7)
and Nude Mice (B, n=5)

pre-injection	3d	1w	2w	3w	4w	5w	6w	7w
(A) 42.9	45.1	53.5	58.6	50.2	41.8	36.5	33.1	34.4
(B) 43.0	55.8	60.0	67.0	59.1	67.4	57.3	64.0	66.3

Example 6
Myoblast Gene Transfer to Correct Anemia Associated with Renal Failure

The current major indication for recombinant human EPO administration is anemia associated with end-stage renal failure (Faulds et al., *Drugs*. 38:863-899 (1989)). Here, the efficacy of a myoblast gene therapy approach is demonstrated using an animal model of renal failure in nude mice. The experiment was designed to determine whether myoblasts can be transplanted and then secrete functional human EPO in an amount sufficient to correct anemia for a long term in these uremic subjects. Transplantation of EPO-producing C2 cells generated marked erythropoiesis as efficiently as in non-uremic mice, indicating that a myoblast gene transfer approach can be applied in renal failure subjects as effectively as in normal subjects. Thus, myoblast gene transfer is means to correct anemia associated with renal failure as well as other types of EPO-responsive anemia.

METHODS

a) Transfection and screening of clones

Human EPO-secreting C2 myoblast clones were prepared as described above. The clones carry the 1.34 kb human EPO cDNA (starting at + 190 nucleotide from the major transcription initiation site to the end of poly A tail) cloned into the plasmid pRC/CMV (Invitrogen, San Diego, CA.). This plasmid bears the cytomegalovirus enhancer/promoter to drive the EPO gene, and a neomycin resistance gene. The highest EPO-producing clone, hereafter called C2-EPO9, produced approximately 33 U/10^6 cells/day of human EPO as determined by radioimmunoassay. The functional activity of EPO produced by this clone was confirmed by an *in vitro* bioassay.

b) Myoblast transplantation

Myoblasts from C2-EPO9 were cultured and harvested as previously described. Under general anesthesia, a total of 4 x 10^7 cells were injected through a 27-gauge needle at 40 different sites (1 x 10^6 cells/10ml/site) of skeletal muscle of both hind limbs in nude mice. Anesthetic agents included 20 mg/kg of ketamine hydrochloride and 3 mg/kg of xylazine hydrochloride (Sigma, St. Louis, MO).

c) Hematocrit measurement

Hematocrit was measured by the microhematocrit method (Koepke, J.A., ed. *Practical Laboratory Hematology*,. 1991, Churchill Livingstone: New York. 112-114). Each week, under general anesthesia, 150 ml of blood was collected by a retroorbital approach into two heparinized capillary tubes. On several occasions, the hematocrit was also measured using a Coulter Counter which showed results parallel to those obtained by the microhematocrit method (not shown).

After hematocrit measurement, serum was recovered from the capillary tubes and stored at -20°C degree for the measurement of EPO concentration and BUN.

d) Creation of renal failure model using nude mice

A renal failure model was created by a two-step nephrectomy (Chanutin et al., *Arch. Intern Med.* 49:767-787 (1932)) using 7-8 week old male nude mice (Charles River Labs., Wilmington, MA). Under general anesthesia using sterile techniques, the right kidney was exposed through a flank incision and decapsulated, and the upper and lower poles (2/3 of the right kidney) were resected. The remnant right kidney was allowed to recover from swelling for a week, and then the total left kidney was resected. The animals were fed standard chow (Harlan Tekland #8656; Harlan Tekland, Madison, WI) containing 24.0% protein and 1.0% phosphorus, and water ad libitum. Renal failure was confirmed by the development of both anemia and uremia. For uremia, blood urea nitrogen (BUN) was determined weekly with a BUN kit (Sigma 535-A; Sigma, St. Louis, MO) using four milliliters of serum.

e) Measurement of serum EPO concentration

Serum concentrations of human EPO were determined by an enzyme linked immunosorbent assay (ELISA) system using a mouse monoclonal antibody according to the manufacturer's protocol (Quantikine IVD; R & D Systems, Minneapolis, MN). This method has a linear range between 2.5 and 200 mU/ml of human EPO with a detection threshold of 0.25 mU/ml.

f) B-galactosidase assays

After euthanasia, skeletal muscle tissue was excised and frozen immediately on dry ice. The excised muscles were then sectioned with a freezing

microtome. The sections were attached to microscope slides, fixed in 0.25% glutaraldehyde for 10 minutes, washed in PBS for 10 minutes, and stained in PBS containing 1 mg/ml of X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$. Sections were incubated at 37°C overnight, rinsed in PBS, mounted, studied under microscope, and photographed.

g) Statistical analysis

Statistical significance was assessed by Student's t-test. $p<0.05$ was taken as significant. Data were expressed as means $\pm$ S.D.

RESULTS

a) Transplantation of EPO-producing myoblasts into normal nude mice

After the transfection with pRC/CMV-EPO, the clones were screened by G418, and 23 clones were randomly selected. Eleven of twenty-three C2 myoblast clones had measurable EPO as determined by RIA ranging from 0.18 to 32.8 U/ml/10^6 cells/day. Using an *in vitro* bioassay with EPO-dependent human leukemic cell line, it was confirmed that the EPO secreted from these engineered muscle cells is functionally active. Transplantation of the cells from C2-EPO9 yielded a marked hematocrit increase for at least three months in healthy normal nude mice, while mice transplanted with parental C2 cells did not show a significant hematocrit change during the period, as summarized in Table 4.

Table 4

Persistent Hematocrit Increase by Transplantation of Human EPO-producing Myoblasts into Non-uremic Nude Mice

Time (weeks)	0	2	4	8	12
control C2 cells (n=6)	44.8 ± 2.4	47.0 ± 3.1	47.2 ± 208	48.8 ± 3.0	49.0 ± 2.3
C2-EPO9 (N=9)	44.6 ± 3.0*	71.2 ± 7.9‡	72.2 ± 7.9‡	67.8 ± 11.8‡	58.0 ± 8.1§

Nude mice were transplanted with 4x107 cells from either control C2 or C2-EPO9, and the microhematocrit was measured weekly by a retroorbital approach.
‡Significantly different from *.
§Significantly different from * and ‡

b) Creation of renal failure model in mice

Ten of twenty-seven nephrectomized mice died within four days after surgery (two after the first, and eight after the second surgery), a mortality rate comparable to a previous report (Gibb et al., *Clinical Immunology and Immunopathology*. 35:276-284 (1985)). Survivors of the acute phase of surgery (17 mice) were followed weekly with hematocrit and BUN determinations. After three weeks, the mice were divided into two groups. Group I included five mice that showed only transient anemia during the three week observation period and were followed without transplantation. Group II included 13 mice that showed persistent anemia with a hematocrit decrease of more than 15% from the preoperative level in three consecutive measurements after the second nephrectomy and were used for transplantation experiments.

In Group II, the mean hematocrit decreased from a preoperative level of 45.2±2.7 to 33.9±3.7 (%0) three weeks after the second nephrectomy. The Group I mice

did not develop further anemia, and the degree of BUN increase was much lower than that of the Group II mice (52.6±13.2 vs. 95.4±16.5 three weeks after the second nephrectomy); presumably due to insufficient nephrectomy.

Among Group II mice, eight mice were transplanted with C2-EPO9 cells, and three mice were followed without transplantation as a non-transplantation control (one mouse died just before transplantation, presumably due to severe uremia). All of the transplanted mice of Group II had a marked hematocrit increase, despite the presence of severe uremia as indicated by the high BUN levels. The rise in hematocrit was comparable to that observed in normal nude mice (Table 4). A mean hematocrit of 68.6±4.2 was achieved two weeks after the transplantation, and this hematocrit increase persisted thereafter. Those without transplantation showed persistent or even deteriorating anemia. All of the Group II mice, except one, died between six and eleven weeks (8.2±1.8 weeks) after the second nephrectomy, while in Group I only one mouse died during the experimental period. The observed survival rate is consistent with previous observations (Kumano et al., *Kidney International*. 30:433-436 (1986)). The one long term survivor in Group I also had the lowest levels of BUN in that group. These data clearly demonstrate the feasibility and potential efficacy of a myoblast gene transfer system even in the face of severe renal failure in mice.

c) Serum EPO level

To examine the secretion of EPO protein from the transplanted cells, serum human EPO concentration was measured using an ELISA. As previously determined, this method did not detect a significant level of

mouse EPO (<2.5 mU/ml) in sera of nude mice phlebotomized (150 ml) weekly over three months (unpublished observation). This observation was confirmed in the non-transplanted renal failure mice in Group II (not shown). Serum EPO measured by this method, therefore, represents just the human EPO produced by the transplanted muscle cells and not endogenous EPO levels. A week after transplantation with C2-EPO9 cells, the serum EPO level was 87.3±22.1mU/ml in group II uremic mice. It declined to 53.8±18.7 mU/m at week 2, and a similar concentration was maintained thereafter until week 8. Thus, the transplanted C2-EPO9 cells persistently produced human EPO at a steady rate for at least two months after transplantation into mice with severe renal failure.

d) <u>The fate of transplanted EPO-secreting myoblasts in renal failure model</u>

To analyze the fate of transplanted myoblasts, C2-EPO9 cells were transduced with BAG retrovirus (Price et al., *Proc. Natl. Acad. Sci. USA*. 84:156-160 (1987)) bearing β-galactosidase and neomycin resistance genes. Since the C2-EPO9 clone had already been maintained in the presence of G418, BAG-transduced clones were selected by positive X-gal staining. Cells from one X-gal positive clone (clone 9-BAG) were expanded and transplanted into nude mice with renal failure according to the same protocol used for C2-EPO transplantation. These mice also showed a marked hematocrit increase as observed in the C2-EPO9 transplanted group II mice (not shown). Six weeks later, X-gal positive myofibers were detected in the entire area of transplantation. At some sites, most of the myofibers were X-gal positive, while at other sites, both X-gal positive and negative myofibers

coexisted. These results demonstrated that the transplanted EPO-secreting myoblasts differentiated by fusing with preexisting host myofibers or themselves and that the transgenes were actively expressed from the transplanted cells for the duration of the experimental protocol.

Discussion

It is unknown how other abnormalities in the uremic syndrome including electrolyte disorders, metabolic acidosis (i.e., glucose intolerance), gastrointestinal disorders, neurologic abnormalities, and metabolic disorders, might affect the outcome of myoblast transplantation. Furthermore, the erythroid response to EPO is significantly reduced, and red blood cell survival is shortened in uremia. In the present experiment, however, it was demonstrated that the transplantation of transformed myoblasts could, even in the face of severe uremia, deliver more than a sufficient amount of human EPO to correct anemia due to renal failure. Furthermore, the therapeutic effects lasted for at least two months. Longer periods of analysis were limited by animal death probably due to severe renal failure. However, with concomitant treatment of renal failure, it is likely the hematocrit increase would persist for more than two months, as was the case with non-uremic mice (Table 4).

The observation that the serum EPO concentration was still high at two months, together with the fact that the half-life of red blood cells in mice is 20-45 days, also supports the likelihood that the increased hematocrit would have been sustained longer than two months, if the uremia had been corrected by dialysis. The sustained high serum human EPO concentration due to transformed myoblast transplantation confirmed that

the observed hematocrit increase was due to human EPO derived from the transplanted cells rather than from endogenous mouse EPO. The persistent presence of X-gal positive myofibers after clone 9-BAG transplantation further supports the notion that the transplanted myoblasts differentiate into myofibers and become a stable source of EPO production in the face of renal failure.

End-stage renal failure patients as well as patients with hypoproliferative anemia secondary to 3'-azido-3'deoxythymidine (AZT) administration are currently treated with 100-150 U of recombinant EPO per kg of body weight per week to maintain a target hematocrit level between 30 and 33, which is equal to 857-1286 U/day for a 60 kg patient. Since C2-EPO9 secreted 32.8 U of EPO/10^6 cells/day, 2.6-3.9 x 10^7 cells would, in theory, be sufficient to provide 1286 U/day. The delivery of this number of muscle cells appears to be feasible, since in a phase I clinical trial of myoblast transfer in Duchenne muscular dystrophy patients, as many as 10^8 myoblasts could be prepared from small muscle biopsy (0.5-1.0g) of first degree relatives and transplanted into patients (Gussoni et al., *Nature*. 356:435-438 (1992)).

The present myoblast gene transfer system could further be optimized for clinical applications. For example, the technique may be modified to include: (1) the use of primary myoblasts and/or (2) the use of an implantable immunoisolation device. Although primary myoblasts might be transfected with EPO cDNA to secrete EPO and increase hematocrit in mice, this approach would require customized preparation of cells for an individual patient to avoid immunorejection. In this regard, a stocked cell line with an immunoisolation device might be a more practical approach for a large population of patients. Within

such a device transformed myoblasts appear to retain an ability to differentiate (Liu et al., *Human Gene Therapy*. 4:291-301 (1993)) and are likely to become a stable source of recombinant protein production.

While mice appear to tolerate the unusually high hematocrit for several months, overproduction of EPO could have potentially deleterious consequences including polycythemia. Although recombinant gene production can be controlled to some degree by the number of cells transplanted, regulated transgene expression could also be achieved, such as by the use of inducible promoters to drive genes of interest, as mentioned above.

The present study demonstrated (1) that myoblast gene transfer technology could correct a disease condition (correction of anemia) as a systemic response to EPO transgene expression, and (2) that myoblast gene transfer is feasible for the delivery of genes of interest (not restricted to EPO) in the setting of severe uremia, a disease condition previously untested for this approach.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: AMGEN INC.
UNIVERSITY OF SOUTHERN CALIFORNIA

(ii) TITLE OF INVENTION: GENE THERAPY VECTOR FOR THE TREATMENT OF LOW OR DEFECTIVE RED BLOOD CELL PRODUCTION

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: AMGEN INC.
(B) STREET: 1840 DEHAVILLAND DRIVE
(C) CITY: THOUSAND OAKS
(D) STATE: CALIFORNIA
(E) COUNTRY: U.S.A.
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1789 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 625..1203

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTCTGG GCTTCCAGAC CCAGCTACTT TGCGGAACTC AGCAACCCAG GCATCTCTGA 60

GTCTCCGCCC AAGACCGGGA TGCCCCCCAG GGGAGGTGTC CGGGAGCCCA GCCTTTCCCA 120

GATAGCACGC TCCGCCAGTC CCAAGGGTGC GCAACCGGCT GCACTCCCCT CCCGCGACCC 180

AGGGCCCGGG AGCAGCCCCC ATGACCCACA CGCACGTCTG CAGCAGCCCC GCTCACGCCC 240

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CGGCGAGCCT CAACCCAGGC CTCCTGCCCC TGCTCTGACC CCGGGTGGCC CCTACCCCTG   300

GCGACCCCTC ACGCACACAG CCTCTCCCCC ACCCCCACCC GCGCACGCAC ACATGCAGAT   360

AACAGCCCCG ACCCCCGGCC AGAGCCGCAG AGTCCCTGGG CCACCCCGGC CGCTCGCTGC   420

GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG GCCACCGCGC CCGCTCTGCT   480

CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG CCCGTGGGGC TGGCCCTGCA   540

CCGCCGAGCT TCCCGGGATG AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT   600

GAGGGACCCC GGCCAGGCGC GGAG ATG GGG GTG CAC GAA TGT CCT GCC TGG       651
                          Met Gly Val His Glu Cys Pro Ala Trp
                            1               5

CTG TGG CTT CTC CTG TCC CTG CTG TCG CTC CCT CTG GGC CTC CCA GTC     699
Leu Trp Leu Leu Leu Ser Leu Leu Ser Leu Pro Leu Gly Leu Pro Val
 10                  15                  20                  25

CTG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG GAG AGG     747
Leu Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg
                 30                  35                  40

TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT ATC ACG ACG GGC TGT GCT     795
Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala
             45                  50                  55

GAA CAC TGC AGC TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT     843
Glu His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val
         60                  65                  70

AAT TTC TAT GCC TGG AAG AGG ATG GAG GTC GGG CAG CAG GCC GTA GAA     891
Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu
     75                  80                  85

GTC TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT GTC CTG CGG GGC CAG     939
Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln
 90                  95                 100                 105

GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG CAG CTG CAT     987
Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His
                110                 115                 120

GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG CTT CGG    1035
Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg
            125                 130                 135

GCT CTG GGA GCC CAG AAG GAA GCC ATC TCC CCT CCA GAT GCG GCC TCA    1083
Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser
        140                 145                 150

GCT GCT CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC    1131
Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe
    155                 160                 165
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CGA GTC TAC TCC AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG    1179
Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly
170                 175                 180                 185

GAG GCC TGC AGG ACA GGG GAC AGA TGACCAGGTG TGTCCACCTG GGCATATCCA   1233
Glu Ala Cys Arg Thr Gly Asp Arg
                190

CCACCTCCCT CACCAACATT GCTTGTGCCA CACCCTCCCC CGCCACTCCT GAACCCCGTC  1293

GAGGGGCTCT CAGCTCAGCG CCAGCCTGTC CCATGGACAC TCCAGTGCCA GCAATGACAT  1353

CTCAGGGGCC AGAGGAACTG TCCAGAGAGC AACTCTGAGA TCTAAGGATG TCACAGGGCC  1413

AACTTGAGGG CCCAGAGCAG GAAGCATTCA GAGAGCAGCT TTAAACTCAG GGACAGAGCC  1473

ATGCTGGGAA GACGCCTGAG CTCACTCGGC ACCCTGCAAA ATTTGATGCC AGGACACGCT  1533

TTGGAGGCGA TTTACCTGTT TTCGCACCTA CCATCAGGGA CAGGATGACC TGGAGAACTT  1593

AGGTGGCAAG CTGTGACTTC TCCAGGTCTC ACGGGCATGG GCACTCCCTT GGTGGCAAGA  1653

GCCCCCTTGA CACCGGGGTG GTGGGAACCA TGAAGACAGG ATGGGGGCTG GCCTCTGGCT  1713

CTCATGGGGT CCAAGTTTTG TGTATTCTTC AACCTCATTG ACAAGAACTG AAACCACCAA  1773

AAAAAAAAAA AAAAAA                                                  1789
```

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 193 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
 1               5                  10                  15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
            20                  25                  30

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
        35                  40                  45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95
```

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
100 105 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
115 120 125

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
130 135 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
145 150 155 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
165 170 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
180 185 190

Arg

CLAIMS

What is claimed is:

1. A method for increasing red blood cell production, comprising the steps of:

transforming muscle cells with a nucleic acid construct comprising an expression control sequence and an erythropoietin gene which is operatively linked to said control sequence, wherein said muscle cells are transformed *in vivo*, or wherein said muscle cells are transformed *in vitro* and subsequently inserted into muscle tissue; and

expressing erythropoietin protein in said transformed muscle cells in an amount sufficient to increase red blood cell production over pretreatment levels.

2. The method according to Claim 1, wherein said expression control sequence comprises a human cytomegalovirus promoter.

3. The method according to Claim 1, wherein said expression control sequence comprises a muscle-specific promoter.

4. The method according to Claim 1, wherein said expression control sequence comprises an inducible promoter.

5. The method according to Claim 1, wherein said muscle cells transformed *in vitro* are placed in an implantable immunoisolation device.

6. A nucleic acid construct for modifying muscle cells to produce erythropoietin, comprising:

a recombinant vector comprising an expression control sequence and an erythropoietin gene which is operatively linked to said expression control sequence, wherein said vector transforms muscle cells to express erythropoietin in an amount sufficient to increase circulating red blood cells.

7. The construct according to Claim 6, wherein said expression control sequence contains an inducible promoter.

8. The construct according to Claim 6, wherein said expression control sequence contains a muscle-specific promoter.

9. A pharmaceutical composition for the modification of muscle cells to produce erythropoietin, comprising:

a nucleic acid construct comprising an expression control sequence and an erythropoietin gene which is operatively linked to said control sequence, wherein upon transfer to a muscle cell said construct elicits the expression of erythropoietin in an amount sufficient to increase circulating red blood cells as compared to pretreatment levels; and

a carrier capable of promoting uptake of said construct by said muscle cells.

10. The composition according to Claim 9, wherein said expression control sequence comprises an inducible promoter.

11. The composition according to Claim 9, wherein said expression control sequence comprises a muscle-specific promoter.

12. Modified muscle cells which expresses an erythropoietin gene following transformation with a nucleic acid construct comprising an expression control sequence and an erythropoietin gene which is operatively linked to said control sequence, wherein said muscle cells are transformed *in vivo*, or wherein said muscle cells are transformed *in vitro* and subsequently implanted, and wherein the modified cells express sufficient erythropoietin to increase the recipient's red blood cell production as compared to pretreatment levels.

13. The method according to Claim 12, wherein said muscle cells transformed *in vitro* are placed in an implantable immunoisolation device prior to implantation.

14. A method for increasing red blood cell production, comprising:

transfecting myoblasts *in vitro* with a recombinant viral vector comprising an expression promoter and an erythropoietin gene which is operatively linked to said promoter thereby forming transfected myoblasts; and

implanting said transfected myoblasts into muscle tissue, wherein said transfected myoblasts fuse to form muscle cells which produce and release erythropoietin at a level sufficient to increase red blood cell production as compared to pretreatment levels.

15. A method for enhancing the production of red blood cells, comprising the steps of:

transforming skeletal muscle cells with a nucleic acid construct comprising an expression control

sequence and an erythropoietin gene which is operatively linked to said control sequence; and

expressing erythropoietin protein in said transformed muscle cells in an amount sufficient to increase red blood cell production.

16. The method according to Claim 15, wherein said expression control sequence comprises an inducible promoter.

17. A genetically modified muscle cell which expresses erythropoietin following the introduction of a nucleic acid construct into said cell, said nucleic acid construct comprising an expression control sequence and an erythropoietin gene which is operatively linked to said control sequence.

18. The modified muscle cell according to Claim 17, wherein said expression control sequence comprises an inducible promoter.

19. The modified muscle cell according to Claim 17, wherein said expression control sequence comprises a transcriptional enhancer element.

FIG. 1

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AAGCTTCTGGGCTTCCAGACCCAGCTACTTTGCGGAACTCAGCAACCCAGGCATCTCTGA
1 ----------+----------+----------+----------+----------+----------+ 60
  TTCGAAGACCCGAAGGTCTGGGTCGATGAAACGCCTTGAGTCGTTGGGTCCGTAGAGACT

  GTCTCCGCCCAAGACCGGGATGCCCCCCAGGGGAGGTGTCCGGGAGCCCAGCCTTTCCCA
61 ----------+----------+----------+----------+----------+----------+ 120
  CAGAGGCGGGTTCTGGCCCTACGGGGGGGTCCCCTCCACAGGCCCTCGGGTCGGAAAGGGT

  GATAGCACGCTCCGCCAGTCCCAAGGGTGCGCAACCGGCTGCACTCCCCTCCCGCGACCC
121 ----------+----------+----------+----------+----------+----------+ 180
  CTATCGTGCGAGGCGGTCAGGGTTCCCACGCGTTGGCCGACGTGAGGGGAGGGCGCTGGG

  AGGGCCCGGGAGCAGCCCCCATGACCCACACGCACGTCTGCAGCAGCCCCGCTCACGCCC
181 ----------+----------+----------+----------+----------+----------+ 240
  TCCCGGGCCCTCGTCGGGGGTACTGGGTGTGCGTCAGACGTCGTCGGGGCGAGTGCGGG

  CGGCGAGCCTCAACCCAGGCCTCCTGCCCCTGCTCTGACCCCGGGTGGCCCCCTACCCCTG
241 ----------+----------+----------+----------+----------+----------+ 300
  GCCGCTCGGAGTTGGGTCCGGAGGACGGGGACGAGACTGGGGGCCCACCGGGGATGGGGAC
```

FIG. 1A

```
                                                                    GCGACCCCTCACGCACACAGCCTCTCCCCCACCCCCGCGCACGCACACATGCAGAT
301 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 360
                                                                    CGCTGGGGAGTGCGTGTGTCGGAGAGGGGGTGGGGGCGCGTGCGTGTGTACGTCTA

                                                                    AACAGCCCCGACCCCCGGCCAGAGCCGCAGAGTCCCTGGGCCACCCCGGCCGCTCGCTGC
361 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 420
                                                                    TTGTCGGGGCTGGGGGCCGGTCTCGGCGTCTCAGGGACCCGGTGGGGCCGGCGAGCGACG

                                                                    GCTGCGCCGCACCGCGCTGTCCTCCCGGAGCCGGACCGGGGCCACCGCGCCCGCTCTGCT
421 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 480
                                                                    CGACGCGGCGTGGCGCGACAGGAGGGCCTCGGCCTGGCCCCGGTGGCGCGGGCGAGACGA

                                                                    CCGACACCGCGCCCCCTGGACAGCCGCCCTCTCCTCTAGGCCCGTGGGGCTGGCCCTGCA
481 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 540
                                                                    GGCTGTGGCGCGGGGGACCTGTCGGCGGAGAGGAGATCCGGGCACCCCGACCGGGACGT

                                                                                                                    B
                                                                                                                    s
                                                                                                                    t
                                                                                                                    E
                                                                                                                    I
                                                                                                                    I
                                                                    CCGCCGAGCTTCCCGGGATGAGGGCCCCCGGTGTGGTCACCCGGCGCGCCCCAGGTCGCT
541 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 600
                                                                    GGCGGCTCGAAGGGCCCTACTCCCGGGGGCCACACCAGTGGGCCGCGCGGGGTCCAGCGA
```

FIG. 1B

```
    GAGGGACCCCGGCCAGGCGCGGAGATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTT
601 ---------+---------+---------+---------+---------+---------+ 660
    CTCCCTGGGGCCGGTCCGCGCCTCTACCCCCACGTGCTTACAGGACGGACCGACACCGAA

                          M  G  V  H  E  C  P  A  W  L  W  L  -

    CTCCTGTCCCTGCTGTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTC
661 ---------+---------+---------+---------+---------+---------+ 720
    GAGGACAGGGACGACAGCGAGGGAGACCCGGAGGGTCAGGACCCGCGGGGTGGTGCGGAG

    L  L  S  L  L  S  L  P  L  G  L  P  V  L  G  A  P  P  R  L  -

    ATCTGTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATC
721 ---------+---------+---------+---------+---------+---------+ 780
    TAGACACTGTCGGCTCAGGACCTCTCCATGGAGAACCTCCGGTTCCTCCGGCTCTTATAG

    I  C  D  S  R  V  L  E  R  Y  L  L  E  A  K  E  A  E  N  I  -

    ACGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAATATCACTGTCCCAGACACCAAA
781 ---------+---------+---------+---------+---------+---------+ 840
    TGCTGCCCGACACGACTTGTGACGTCGAACTTACTCTTATAGTGACAGGGTCTGTGGTTT

    T  T  G  C  A  E  H  C  S  L  N  E  N  I  T  V  P  D  T  K  -
```

FIG. IC

```
                                                    GTTAATTTCTATGCCTGGAAGAGGATGGAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAG
841 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 900
                CAATTAAAGATACGGACCTTCTCCTACCTCCAGCCCGTCGTCCGGCATCTTCAGACCGTC

                V  N  F  Y  Y  A  W  K  R  R  M  E  E  V  G  G  Q  Q  A  A  V  E  E  V  W  Q  -

                GGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCCAGGCCCTGTTGGTCAACTCTTCC
901 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 960
                CCGGACCGGGACGACAGCCTTCGACAGGACGCCCCCGGTCCGGGACAACCAGTTGAGAAGG

                G  L  A  L  L  S  E  A  V  L  R  G  Q  Q  A  L  L  V  N  S  S  -

                CAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTCAGTGGCCTTCGCAGCCTC
961 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1020
                GTCGGCACCCTCGGGGACGTCGACGTACACCTATTTCGGCAGTCACCGGAAGCGTCGGAG

                Q  P  W  E  P  L  Q  L  H  V  D  K  A  V  S  G  L  R  S  L  -

                ACCACTCTGCTTCGGGCTCTGGGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATGCGGCC
1021 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1080
                TGGTGAGACGAAGCCCGAGACCCTCGGGTCTTCCTTCGGTAGAGGGGAGGTCTACGCCGG

                T  T  L  L  R  A  L  G  A  Q  K  E  A  I  S  P  P  D  A  A  -

                TCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTAC
1081 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1140
                AGTCGACGAGGTGAGGCTTGTTAGTGACGACTGTGAAAGGCGTTTGAGAAGGCTCAGATG

                S  A  A  P  L  R  T  I  T  A  D  T  F  F  R  K  L  F  R  V  Y  -
```

FIG. 1D

```
                                                  TCCAATTTCCTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCAGGACAGGGGAC
1141 ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1200
                AGGTTAAAGGAGGCCCCTTTCGACATGTGTCCCCTCCGGACGTCCTGTCCCCTG

                S  N  F  L  R  G  K  K  L  K  L  Y  T  G  E  A  C  R  T  G  D  -

                AGATGACCAGGTGTGTCCACCTGGGCATATCCACCACCTCCCTCACCAACATTGCTTGTG
1201 ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1260
                TCTACTGGTCCACACAGGTGGACCCGTATAGGTGGTGGAGGGAGTGGTTGTAACGAACAC

                R  *

                CCACACCCTCCCCCGCCACTCCTGAACCCCGTCGAGGGCTCTCAGCTCAGCGCCAGCCT
1261 ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1320
                GGTGTGGGAGGGGGCGGTGAGGACTTGGGGCAGCTCCCCGAGAGTCGAGTCGCGGTCGGA

                GTCCCATGGACACTCCAGTGCCAGCAATGACATCTCAGGGGCCAGAGGAACTGTCCAGAG
1321 ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1380
                CAGGGTACCTGTGAGGTCACGGTCGTTACTGTAGAGTCCCCGGTCTCCTTGACAGGTCTC
```

FIG. 1E

```
     AGCAACTCTGAGATCTAAGGATGTCACAGGGCCAACTTGAGGGCCCAGAGCAGGAAGCAT
1381 ---------+---------+---------+---------+---------+---------+ 1440
     TCGTTGAGACTCTAGATTCCTACAGTGTCCCGGTTGAACTCCCGGGTCTCGTCCTTCGTA

     TCAGAGAGCAGCTTTAAACTCAGGGACAGAGCCATGCTGGGAAGACGCCTGAGCTCACTC
1441 ---------+---------+---------+---------+---------+---------+ 1500
     AGTCTCTCGTCGAAATTTGAGTCCCTGTCTCGGTACGACCCTTCTGCGGACTCGAGTGAG

     GGCACCCTGCAAAATTTGATGCCAGGACACGCTTTGGAGGCGATTTACCTGTTTTCGCAC
1501 ---------+---------+---------+---------+---------+---------+ 1560
     CCGTGGGACGTTTTAAACTACGGTCCTGTGCGAAACCTCCGCTAAATGGACAAAAGCGTG

     CTACCATCAGGGACAGGATGACCTGGAGAACTTAGGTGGCAAGCTGTGACTTCTCCAGGT
1561 ---------+---------+---------+---------+---------+---------+ 1620
     GATGGTAGTCCCTGTCCTACTGGACCTCTTGAATCCACCGTTCGACACTGAAGAGGTCCA

     CTCACGGGCATGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACACCGGGGTGGTGGGAA
1621 ---------+---------+---------+---------+---------+---------+ 1680
     GAGTGCCCGTACCCGTGAGGGAACCACCGTTCTCGGGGGAACTGTGGCCCCACCACCCTT

     CCATGAAGACAGGATGGGGGCTGGCCTCTGGCTCTCATGGGGTCCAAGTTTTGTGTATTC
1681 ---------+---------+---------+---------+---------+---------+ 1740
     GGTACTTCTGTCCTACCCCCGACCGGAGACCGAGAGTACCCCAGGTTCAAAACACATAAG

     TTCAACCTCATTGACAAGAACTGAAACCACCAAAAAAAAAAAAAAAAAA
1741 ---------+---------+---------+---------+--------- 1789
     AAGTTGGAGTAACTGTTCTTGACTTTGGTGGTTTTTTTTTTTTTTTTTT
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/13066

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C07K14/505 A61K48/00 C12N5/10 C12N15/85
C12N15/86 A61K38/22 A61K9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,88 00241 (UNIV WASHINGTON) 14 January 1988 see page 1, line 13 - page 2, line 5 see examples ---	1-19
Y	WO,A,93 03768 (UNIV LELAND STANFORD JUNIOR) 4 March 1993 see page 9, line 17 - page 14, line 5 see page 17, line 19 - page 19, line 22 --- -/--	1-4, 6-12, 14-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 1995	03. 03. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/13066

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUMAN GENE THERAPY, vol. 4, no.3, June 1993 pages 291-301, LIU, H.-W. ET AL. 'Expression of human factor IX by microencapsulated recombinant fibroblasts' cited in the application see page 293, left column, line 21 - right column, line 5	5,13
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, November 1992 WASHINGTON US, pages 10892-10895, DAI, Y. ET AL. 'Gene therapy via primary myoblasts: long term expression of factor IX protein following transplantation in vivo' see the whole document	1,2,4,6, 7,9,10, 12,14-19
A	HUMAN GENE THERAPY, vol. 2, no.1, 1991 pages 15-26, SALMINEN, A. ET AL. 'Implantation of recombinant rat myocytes into adult skeletal muscle: a potential gene therapy' cited in the application	
A	SOMATIC CELL MOL GENET 19 (2). 111-122, March 1993 PRIGOZY, T. ET AL. 'DIRECT DNA INJECTION INTO MOUSE TONGUE MUSCLE FOR ANALYSIS OF PROMOTER FUNCTION IN-VIVO.' see figure 1	
A	SCIENCE, vol. 254, 6 December 1991 LANCASTER, PA US, pages 1509-1512, DHAWAN, J. ET AL. 'Systemic delivery of human growth hormone by injection of genetically engineered myoblasts'	
T	WO,A,94 01129 (SALK INST FOR BIOLOGICAL STUDI) 20 January 1994 see page 3, line 10 - page 7 see figure 1 see claims	1-19

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/ 13066

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-5 and 12-19 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International application No.
PCT/US 94/13066

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8800241	14-01-88	AU-B-	611088	06-06-91
		AU-A-	7475787	07-01-88
		DE-A-	3779206	25-06-92
		EP-A,B	0255231	03-02-88
		JP-A-	63126488	30-05-88
WO-A-9303768	04-03-93	AU-A-	2515792	16-03-93
		NO-A-	940597	19-04-94
WO-A-9401129	20-01-94	CA-A-	2137456	20-01-94